

ISOLATION OF XYLAN DEGRADING MICROORGANISM FROM LOCAL
HOT SRING

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of the requirements for the award of the degree of
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“I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor In Chemical Engineering (Biotechnology)”

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Date : April 2008

DECLARATION

I declare that this thesis entitled “Isolation of xylan degrading microorganism from local hot spring” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature :.....

Name of Candidate : Mohd Nizam B Zakariya

Date : April, 2008

DEDICATION

*Special Dedication to my family members that always love me,
My friends, my fellow colleague
and all faculty members*

For all your Care, Support and Believe in me.

*Sincerely
Mohd Nizam B Zakariya*

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ABSTRACT

The purpose of this study was to isolate xylan-degrading microorganisms from local hot spring. The samples were taken from a local hot spring at Sungai Klah, Perak. They were screened for the best xylan degrader by using selective culture media. Then, the xylan degrader microorganism was characterized by using four staining method which were gram staining, acid-fast staining, simple staining and spore staining. The morphology of the microorganism was observed after all the staining was done by observing the changes of the staining colour. Based on the results, the microorganism was in rod shaped for simple staining, gram negative for gram staining, positive results for both spore and acid-fast staining. In conclusion, this staining method can be used to characterize the microorganism based on general information, so the microorganism was probably in genus *Bacillus*.

ABSTRAK

Tujuan kajian ini adalah untuk mengasingkan mikroorganisma yang boleh menguraikan xylan daripada kolam air panas tempatan. Sampel diambil daripada kolam air panas tempatan yang terletak di Sungai Klah, Perak. Mikroorganisma tersebut diasingkan dengan menggunakan kultur media terpilih. Kemudian, mikroorganisma itu dicirikan melalui empat kaedah 'staining' iaitu 'simple staining', 'gram staining', 'spore staining', dan 'acid-fast staining'. Morfologi mikroorganisma tersebut dapat dilihat setelah 'staining' dilakukan dengan melihat perubahan warna 'staining' tersebut. Hasil daripada kajian ini didapati mikroorganisma tersebut adalah berbentuk rod untuk 'simple staining', gram negatif untuk 'gram staining', positif untuk 'spore' dan 'acid-fast staining'. Kesimpulannya, kaedah 'staining' dapat digunakan untuk mengelaskan mikroorganisma secara kasar, oleh itu mikroorganisma itu mungkin terdiri daripada genus *Bacillus*.

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LIST OF SYMBOLS

%	-	percentage
g	-	gram
h	-	hours
kDa	-	kilo Dalton
L	-	liter
M	-	molar
min	-	minutes
ml	-	milliliters
°C	-	degree Celsius
rpm	-	rotation per minutes
s	-	second
v	-	volume
w	-	weight

CHAPTER 1

INTRODUCTION

1.1 Introduction

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They can be divided into three groups: cellulose, hemicellulose and pectin. Cellulose represents the major constituent of cell wall polysaccharides and consists of a linear polymer of β -1,4-linked D-glucose residues. The cellulose polymers are present as ordered structures (fibres) and their main function is to ensure the rigidity of the plant cell wall. Hemicelluloses are more heterogeneous polysaccharides and are the second most abundant organic structure in the plant cell wall. The major hemicellulose polymer in cereals and hardwood is xylan. Xylan consists of a β -1,4-linked D-xylose backbone and can be substituted with different side-groups such as L-arabinose, D-galactose, acetyl, feruloyl and p-coumaroyl and glucuronic acid residues (Wilkie and Woo, 1977). A second hemicellulose structure commonly found in soft and hardwoods is (galacto)glucomannan (Timell, 1967), which consists of a backbone of β -1,4-linked mannose and glucose residues and contains galactose side groups. Softwoods contain mainly galactoglucomannan whereas in hardwoods glucomannan is the most common form.

The xylan structure, however, can differ greatly depending on its origin (Huisman *et al.*, 2000). Basically, a xylan structure consists of D-xylopiranose units, linked by β -1,4 bonds, which can be exhibited either in linear or branched form (Bastawde, 1992). A complete and efficient enzymatic hydrolysis of this complex polymer depends mainly on two types of enzymes: endo-1,4- β -xylanases

(1,4- β -D-xylohydrolase; EC 3.2.1.8), which hydrolyze the xylanopyranose of the central chain, and the β xylosidases (1,4- β -D-xylano xylohydrolase; EC 3.2.1.37), which hydrolyze xylobiose and other xylooligosaccharides resulting from the action of endoxylanases. Other enzymes such as acetylxylan esterases, α -glucuronidase, and α -L-arabinofuranosidase act in synergism with xylanases to degrade specific groups (Beg *et al.*, 2001).

Xylanase is one of the microbial enzymes that has aroused great interest recently due to its biotechnological potential in many industrial processes such as in xylitol and ethanol production (Beg *et al.*, 2001), in the cellulose and paper industry (Wong *et al.*, 1988), in the production of oligosaccharides (Pellerin *et al.*, 1991), to obtain cellular proteins, liquid fuels, and other chemical substances (Biely, 1985), in the food industry (Haltrich *et al.*, 1996), and in poultry, pork, and caprine feeding (Simoes and Tauk-Tornisielo, 2006). Due to industrial purpose, the more stable enzymes are in high demand, leading to various researches done on producing thermostable xylanase from thermophilic and hyperthermophilic microorganism. Xylanases are extracellular enzymes produced by microorganisms such as bacteria (saprophytic and phytopathogenous), mycorrhizic fungi, and some yeasts. The enzyme is also found in protozoa, insects, crustaceans, snails, seaweed, and also seeds of plants during the germination phase in the soil (Wong *et al.*, 1988). Xylanases catalyze xylan hydrolysis, the major hemicellulose component in plant cell walls.

As enzymes produced from thermophilic microorganism gain a great attention in many areas, so the purpose of this study is to find and explore the potential of thermophilic microorganism in producing thermostable enzyme, especially xylanase enzyme. The production of thermostable xylanase from local isolates can be used in xylan-processing industry which can increase the reaction rate and reduce the energy consumption as well as production cost. Besides, this study also utilizes the local source of xylan degrading especially from local hot spring.

1.2 Problem Statement

Since the incoming pulp for enzymatic bleaching is hot and alkaline, the use of thermostable alkaline xylanases is very attractive from economical and technical point of view. As a result, the search for novel xylanases for pulp and paper industries has continued (Mamo *et al.*, 2006). Nowadays, we can see a lot of research done by scientist to study the morphology of xylanase. Due to industry application, this enzyme give a lot of advantage such as increasing the reaction rate and reduce the energy consumption as well as cost production. Besides, this studies also to utilize local source of xylan degrading especially from local hot spring.

1.3 Objectives

To isolate xylan-degrading microorganisms from a local hot spring.

1.4 Scopes of study

The scopes of this study are as follows:

- a. To isolate the microorganism from a local hot spring
- b. To screen the microorganism from xylan degraders
- c. To carry out the morphology study of microorganism

CHAPTER 2

LITERATURE REVIEW

2.1 Thermophiles

Thermophile is an organism with a growth temperature optimum between 45°C and 80°C. During the past decades, the interest in thermophilic microorganisms, which live and thrive at temperatures up to 100 °C, has been increasing. Thermophilic bacteria were first isolated in 1879 by Miquel J.C, who found bacteria capable of growing at 72 °C. Thermophiles have been isolated from a variety of hot environments such as terrestrial and submarine geothermal areas, hot oil-field production waters, deep subterranean cores, hot spring and from biologically self-heated materials. Thermophilic microorganisms are known to be a source of thermostable hydrolytic enzymes (Sunna *et al.*, 1996). Enzymes from thermophilic microorganisms are generally thermostable and also stable in presence of denaturing agents and organic solvents. Many of these enzymes find wide industrial use in food and feed, paper and pulp, modification of complex polysaccharides and in organic biosynthesis.

2.2 Xylan

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall (Timell, 1967). It is a heteropolymer with backbone of β -1,4- d-xylanopyranosyl residues and branches of neutral or uronic monosaccharides and oligosaccharides (Joseleau *et al.*,1992). Xylan is a heteropolymer composed primarily of β -1,4-linked xylose with various amounts of arabinose, glucose, galactose, uronic acids, and other sugars as sidegroups, depending on the plant source. Xylan is widely distributed in plant cell walls and forms a main part of the hemicellulose fraction. In some higher plants and agricultural wastes, xylan constitutes from 20-40% of the dry weight (Rifaat *et al.*, 2005a). Xylan together with hemicellulose forms the second most abundant renewable polysaccharide in the biosphere.

2.2.1 Structure features of xylan

The structure of xylans found in cell walls of plants can differ strongly depending on the origin, but always contains a β -1,4-linked xylose backbone (Wilkie, 1979). As shown in Figure 2.1, the schematic representation of xylan, also lists the different structures which can be attached to the xylan backbone and which cause the large variety of xylan structures found in plants. Although most xylans are branched structures, some linear polysaccharides have been isolated (Montgomery *et al.*, 1956; Eda *et al.*, 1976). Cereal xylans contain large quantities of L-arabinose and are therefore often referred to as arabinoxylans, whereas hardwood xylans are often referred to as glucuronoxylans due to the high amount of glucuronic acid attached to the backbone.

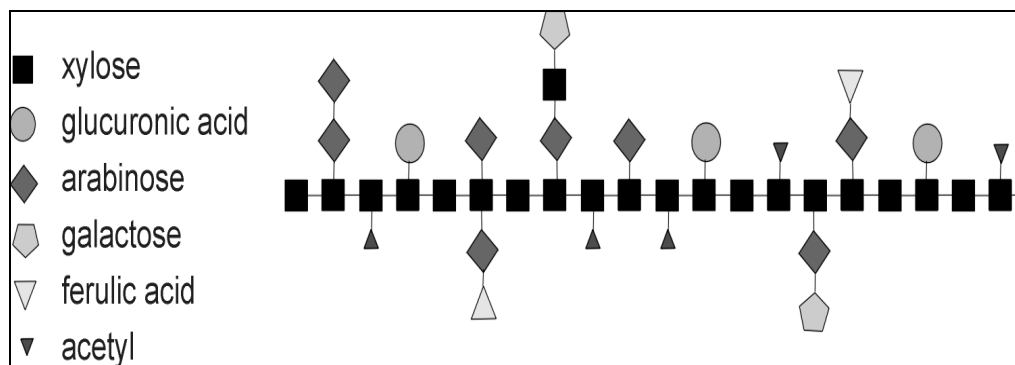


Figure 2.1: Structure of the xylan (Ronald and Visser, 2001).

Arabinose is connected to the backbone of xylan via an α -1,2- or α -1,3-linkage as single residues or as short side chains. These side chains can also contain xylose, β -1,2-linked to arabinose, and galactose which can be either β -1,5-linked to arabinose or β -1,4-linked to xylose. Acetyl residues are attached to O2 or O3 of xylose in the backbone of xylan, but the degree of acetylation differs strongly amongst xylans from different origin. Glucuronic acid and its 4-O-methyl ether are attached to the xylan backbone via a α -1,2-linkage, whereas aromatic (feruloyl and *p*-coumaroyl) residues have so far only been found attached to O5 of terminal arabinose residues. As a consequence of all these features the xylans form a very heterogeneous group of polysaccharides (Brillouet and Joseleau, 1987; Bajpai, 1997; Schooneveld- Bergmans *et al.*, 1998).

2.3 Xylanase

Xylanases (endo- 1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) degrade the xylan backbone into small oligomers. These enzymes are required for many applications such as bleaching of Kraft pulp, increasing the brightness of pulp, improving the digestibility of animal feed and for clarification of fruit juices (Biely *et al.*, 1985). Due to industrial purpose, the more stable enzymes are in high demand, leading to various researches done on producing thermostable xylanase from thermophilic and hyperthermophilic microorganism. Xylanases are extracellular enzymes produced by microorganisms such as bacteria (saprophytic and

phytopathogenous), mycorrhizic fungi, and some yeasts. The enzyme is also found in protozoa, insects, crustaceans, snails, seaweed, and also seeds of plants during the germination phase in the soil (Wong *et al.*, 1988). Xylanases catalyze xylan hydrolysis, the major hemicellulose component in plant cell walls. Xylanases can be grouped into two families, F and G, based on hydrophobic cluster analysis and sequence homology. Of these, the family F xylanases are somewhat larger and have a molecular mass of approximately 35 kDa, while family G xylanases have a molecular mass of only about 20 kDa. Families F and G correspond to families 10 and 11 in the numerical classification of glycosyl hydrolases (Krengel and Dijkstra, 1996)

Xylanases are typical endo acting enzyme and attack the xylan chain in a random manner, causing a decrease degree polymerization of the substrate and liberating shorter oligomers, xylooligosaccharides and even xylose. The mode of action of different xylanases and hydrolysis products vary according to the source of the enzyme.

Xylanases belong to the glucanase enzyme family and are characterized by their ability to break down various xylans to produce short-chain xylooligosaccharides. Xylanase readily crystallizes in ammonium sulfate and sodium potassium phosphate across pH 3.5 to 9.0. Xylanase can also be crystallized with other salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulfate. Xylanase solubility in phosphate buffer (pH 9) decreases in the temperature range of 0 to 10°C but remains constant in the range of 10 through 37°C. Xylanase has been extracted from many different fungi and bacteria. It is commonly used in animal feeds, paper production, and food production (Krengel and Dijkstra., 1996). Xylanase is a hydrolase that catalysis of complex sugar primarily xylan and certain related compound to simple sugar the primary product being xylose (Rengasayee *et al.*, 2005). Table 2.1 summarizes the characteristic of xylanase.

Table 2.1: Characteristics of xylanase (Rengasayee *et al.*, 2005).

	Characteristic
1) Systematic name	1,4- β -D-xylanohydrolase
2) Recommended name	Endo-1,4- β -xylanase
3) Type	Hydrolase
4) Substrate	Xylan
5) Product	Xylose
6) Sources	- Bacterial: <i>Bacillus polymyxa</i> , <i>Cryptococcus albidus</i> - Fungal: <i>Aspergillus</i> spp.(<i>nidulans</i> , <i>Ochraceus, fumugatus</i>), <i>Trichoderma</i> spp.
7) pH range	Acidic (3.5-7.0)
8) Optimum pH	5.3
9) Temperature range	35-60°C
10) Optimum temperature	55°C

Over the last few decades, there has been a growing interest in lignocellulose bioconversion as a renewable energy source. Xylan is the major constituent of hemicellulose and has a high potential for degradation to useful end products. Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side product generation. Xylanases have found applications in the food, feed and pulp and paper industries. Xylanases are of special significance to the pulp and paper industry, where they reduce the amount of chlorine and chlorine dioxide used for bleaching paper pulp. Xylanase pretreatment has been reported to lower bleaching chemical consumption and to result in greater final brightness. Since pulp-bleaching processes are carried out at high temperature and under alkaline conditions, thermostable and alkali-tolerant xylanases are well suited for such industrial processes. The availability of xylanases isolated from nature with the desired thermostability and pH characteristics is limited but the potential benefits of using these enzymes for

biotechnological processes has encouraged widespread research endeavours towards producing desirable xylanases through protein engineering using techniques such as site-directed mutagenesis (Stephens *et al.*, 2007).

2.3.1 Xylan hydrolysis reaction

β -3-1,4-Xylans are heterogeneous polysaccharides found in the cell walls of all land plants and in almost all plant parts. The hydrolysis of their characteristic backbone, consisting of β -1,4-linked D-xylosyl residues, involves P-1,4- xylanases (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) and β -xylosidases (1,4- β -D-xylan xylohydrolase; EC 3.2.1.37). In general terms, the xylanases attack internal xylosidic linkages on the backbone and the β -xylosidases release xylosyl residues by endwise attack of xylooligosaccharides. Although many xylanases are known to release xylose during the hydrolysis of xylan or xylooligosaccharides, xylobiase activity has only been reported in β -xylosidases. These enzymes are the major components of xylanolytic systems produced by biodegradative microorganisms such as fungi and bacteria, whose activities are important for the maintenance of carbon flow in the carbon cycle and thus biomass turnover in nature (Ken *et al.*, 1988). Figure 2.2 illustrates the xylan hydrolysis.

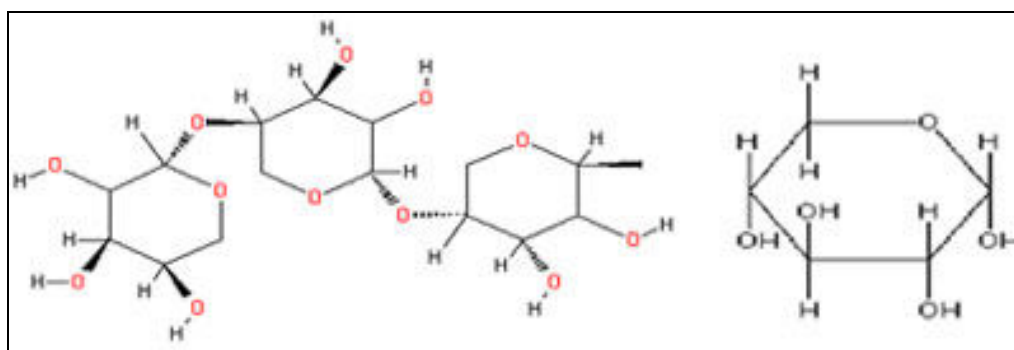


Figure 2.2: Hydrolysis of xylan (Krengel and Dijkstra., 1996).

2.3.2 Xylanase producer

Multiple xylanases have been reported in numerous microorganisms. Five different xylanases have been purified from the culture filtrate of *Aspergillus niger* 11 and from Rhozyme, a crude enzyme from *A. niger*. Other workers have purified at least three xylanases from *Clostridium stercorarium*, *Streptomyces* sp. strain 3137, *Streptomyces exfoliatus* MC₁, *Trichoderma harzianum* E58, *Trichoderma reesei* QM9414, *Aeromonas* sp. strain 212, *Penicillium janthinellum*, and *Talaromyces byssochlamydoides* YH-50 (Ken *et al.*, 1988). The extent of xylanase multiplicity in microorganisms remains to be answered, particularly since a zymogram technique has detected five major and ten minor xylanases in the culture filtrate of *A. niger* 14 and three major and ten minor xylanases in Cellulysin, a commercial enzyme from *Trichoderma viride*. Extensive xylanase multiplicity in Cellulysin had also been reported by other workers (Ken *et al.*, 1988). Table 2.2 shows the characteristic of thermostable xylanase producer.

Table 2.2: Thermostable xylanase producer (Haki and Rakhshit, 2003).

Organism	Optimal Temperature (°C)	Optimal pH
<i>Aspergillus</i> sp.	60	5.0
<i>Aspergillus niger</i>	45-50	4.5-5.0
<i>Bacillus amyloliquefaciens</i>	80	6.8-7.0
<i>Bacillus circulans</i>	80	6.0-7.0
<i>Bacillus</i> sp.	60-75	8.0-9.0
<i>Bacillus</i> sp. strain SPS-0	75	6.0
<i>Bacillus subtilis</i>	50	6.0
<i>Clostridium abosum</i>	75	8.5
<i>Dictyoglomus</i> sp. Strain B1	90	6.0-7.0
<i>Fusarium proliferation</i>	55	5.0-5.5
<i>Pyrococcus furiosus</i>	100	6.0
<i>Pyrococcus furiosus</i>	102	-
<i>Scytalidium thermophilum</i>	65	6.0
<i>Streptomyces</i> sp. Strain S38	60	6.0
<i>Sulfolobus solfataricus</i>	105	5.3
<i>Tekeromyces lanuginosus</i>	60-70	6.7-7.0
<i>Tekeromyces lanuginosus</i> -SSBP	70-75	6.5
<i>Thermoascus aurantiacus</i>	50	5.0
<i>Thermotoga maritima</i> MSB8	92	6.2
<i>Thermotoga neapolitana</i>	95	6.0
<i>Thermotoga thermarum</i>	80	6.6

2.3.3 Application of xylanase

Xylanases are involved in the degradation of complex plant cell wall polysaccharides. Plant cell walls are a major part of the crude biomass which is used in a wide variety of industrial processes. A first step in the industrial processing of biomass frequently involves (partial) degradation of the polymeric fraction. It is therefore obvious that enzymes capable of degrading the plant cell wall can be applied in many of these processes and provides a good alternative to chemical processing. In this section, examples of industrial applications of plant cell wall-degrading enzymes are given. Applications of xylanolytic enzymes can be found in a variety of industrial processes such as kraft pulp bleaching, de-inking of newsprint, poultry industry, wine production, baking and forage digestions (Rengasayee *et al.*, 2005)

2.3.3.1 Kraft pulp bleaching

Xylanase is used in paper industry to improve the strength of cellulose fibers in bleached Kraft Pulp (from bamboo and eucalyptus). It improves pulp fibrillation and water retention, reduces beating times in virgin pulps, restores bonding, increases freeness in recycled fibers and selectively removes xylan from dissolving pulps (Rengasayee *et al.*, 2005).

Pulp for paper and paperboard production also is conventionally processed by Kraft pulping. In this process, free cellulose fibers are obtained by dissolving the cementing lignin in alkaline cooking solution. The resulting pulp contains residual lignin and lignin derivatives, which can be covalently attached to carbohydrate moieties and give undesirable brownish colour to the pulp. The removal of this characteristic coloration is done by a multistage bleaching process, which involves elemental chlorine. Although chlorine-based bleaching of pulp is effective, it results in chlorinated organic by-products which are reported to have highly persistent toxic and mutagenic effects. Because of the growing public concern about environment